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14. ABSTRACT

The goal of this project is to develop novel therapies for breast cancer based on the oncolytic virus, vesicular stomatitis virus (VSV). Studies have shown that matrix (M) protein mutants of VSV, such as rM51R-M virus, are excellent candidates for anti-tumor therapies due to the ability of these viruses to target and kill tumor cells, while sparing normal cells. However, not all tumors are amenable to VSV treatments in vivo. In data presented here, we determined that normal mammary cells are more resistant to VSV-induced cytopathic effect than breast cancer cells. However, in syngeneic breast cancer system in vivo, rM51R-M virus is only partially effective at killing breast tumors derived from 4T1 cells. Our results indicate that the immune response may be attenuating the replication and spread of this virus at the tumor site. To enhance the ability of rM51R-M virus to selectively target and kill tumor cells, we carried out a combination treatment together with the anti-tumor cytokine, IL-12. Our data indicate that rM51R-M virus alone was as effective as IL-12 and the combination therapy at inducing an immune response during tumor therapies. Furthermore, the combination therapy was as effective as single treatments at partially controlling the growth of the primary tumor. However, it appeared to be slightly more effective at treating metastatic tumors. In conclusion, although enhancing the immune response delays tumor growth, none of these therapies were able to completely eliminate the existing tumor. It is possible that further enhancing the immune system may be helpful in overcoming suppressive tumor mechanisms.

15. SUBJECT TERMS
vesicular stomatitis virus, interleukin 12, breast cancer, interferon

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INTRODUCTION:

The goal of this project is to develop novel therapies for breast cancer based on the oncolytic virus, vesicular stomatitis virus (VSV). VSV is currently being developed as an oncolytic virus for the selective treatment of several types of cancers (1-3, 5, 7, 9, 10). Previous studies have shown that VSV kills tumor cells more effectively than many normal cell types due to defects in the antiviral response (including IFN) in tumor cells (3, 9). However, our preliminary studies indicate that several tumor cell lines, including human breast cancer cells, are differentially susceptible to killing by VSV. Experiments proposed here seek to determine whether human breast cancer cells and murine breast cancer cell lines that produce metastatic tumors *in vivo* show differential susceptibility to VSV-induced cell killing. Furthermore, we plan to investigate whether the efficacy of VSV therapies can be enhanced by co-treatment with an anti-tumor cytokine, IL-12. Our hypothesis is that we will enhance killing of tumors that are more resistant to virus infection, as well as those that are highly sensitive to infection.

BODY:

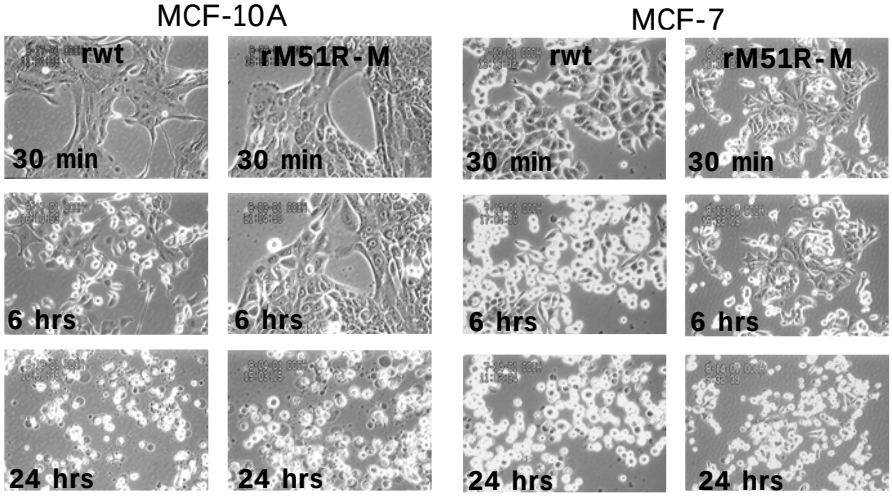
The previous goal of this proposal was to engineer a virus expressing IL-12 to treat breast cancer cells in vivo. However, we modified this goal and received a no-cost extension due to several difficulties that were encountered. First of all, we had a difficult time cloning the IL-12 gene within the VSV genome. Although this has been done with other cytokines, it proved to be more problematic with IL-12. During this work, we also found that another group published a paper in which they used a virus very similar to our M51R-M mutant virus to treat 4T1 cells in vitro (4). This group found that the M51R-M mutant was only partially effective at treating this particular tumor type. 4T1 cells are poorly immunogenic and thus, induce weak tumor-specific immune responses. Therefore, it is possible that these tumor cells grow more aggressively and are thus more difficult to treat with VSV. Therefore, we wanted to ask whether the immune response was aiding, or hindering treatment with VSV. This is a critical question in the oncolytic virus field but few investigators have investigated the role of the immune response during tumor therapies. Furthermore, we wanted to determine whether addition of IL-12 naked DNA would enhance tumor treatment. Thus, we obtained a plasmid from David Mahvi at University of Wisconsin-Madison that expresses IL-12 in vitro and in vivo and has been used to treat tumors in vivo (8). We reasoned that we could better control levels of IL-12 expression from plasmid DNA than that expressed from the virus. This would provide an advantage for therapies in which there exists a fine balance in the ability of the immune response to aid in therapies without eliciting a deleterious inflammatory response. In addition to these studies, we first wanted to ask how individual breast cancer cells versus normal cells respond to infection with VSV.

<u>Aim1: To determine whether human breast cancer cells and a murine breast cancer cell line that produces metastatic tumors *in vivo* (4T1) show differential susceptibility to VSV-induced cell killing (month 1-6)</u>.

Task1: Determine the ability of a wt strain of VSV (rwt virus) and an M protein mutant virus (rM51R-M) to kill nontumorigenic cells versus breast cancer cells in vitro.

M protein mutants of VSV are being designed as safer, and effective, vectors for tumor therapies due to their inability to suppress the type I IFN response (1, 9). Therefore, the idea is that these viruses enhance IFN activity in normal cells which serves to attenuate virus replication and spread in normal tissues, while retaining the ability to target and kill tumor cells. To test this hypothesis, we determined the ability of a wt virus (rwt) and an M protein mutant of VSV (rM51R-M) to kill MCF7 breast cancer cells as compared to MCF10A nontumorigenic mammary cells. Cells were infected with rwt and rM51R-M viruses at a multiplicity of 10pfu/cell. At various times post infection, phase contrast images were taken illustrating the cytopathic effects of VSV in these cells

Figure 1: Nontumorigenic MCF-10A cells are more resistant to the cytopathic effects induced by VSV than MCF-7 cells.



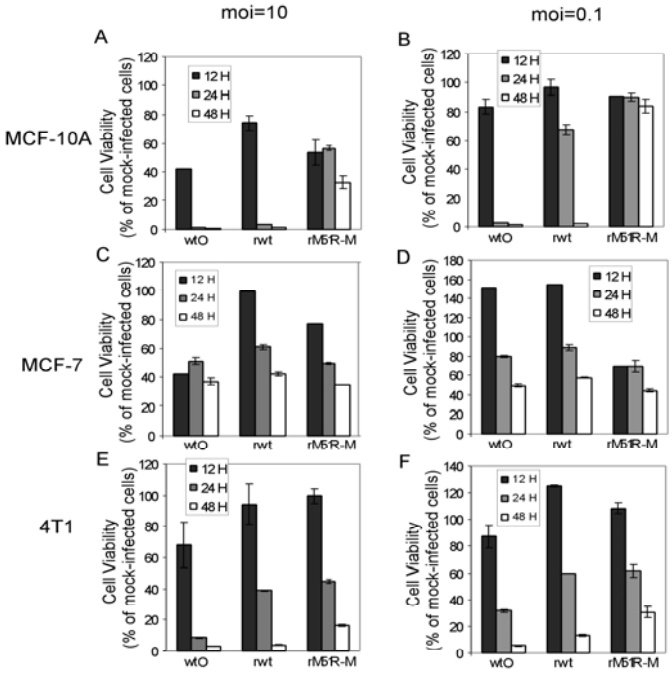
As shown in figure 1, both rwt and rM51R-M virus effectively killed MCF7 breast cancer cells starting as early as 30 minutes post-infection, as indicated by the rounding of cells characteristic of VSV infection. By 6 hours post-infection, most cells were rounded. In contrast, MCF10A nontumorigenic cells were more resistant to VSV-induced cytopathic effect, especially that of rM51R-M virus.

To quantitate the ability of VSV to kill these cells, we carried out an MTT assay to measure the metabolic activity in infected cells (Figure 2). MCF10A, MCF7 and a mouse breast cancer cell line, 4T1, were infected with rwt and rM51R-M viruses at MOIs of 10 and 0.1 pfu/cell. As a control, cells were infected with a naturally occurring wt strain of VSV (wtO) that exhibits cytopathic effect both in vivo and in vitro. The lower MOI (0.1) measures the ability of virus to spread to surrounding cells and exert it cytopathic effect, whereas the higher MOI determines the ability of cells to die in a synchronous virus infection.

At both MOIs (10 and 0.1pfu/cell), MCF10A cells were more resistant to infection with rM51R-M virus than the wt strains of VSV, wtO and rwt viruses (Figure 2). For example at 24-48 hours post-infection, while most of cells infected with rwt and wtO viruses died, 40-80% of rM51R-M virus-infected cells remained viable. This is presumably due to the ability of these cells to mount an effective IFN response to the virus infection. In contrast, rwt and rM51R-M virus were equally effective at killing MCF7 breast cancer cell lines. In addition, the mouse breast cancer

cell line, 4T1, was also susceptible to killing by VSV since by 48hours post-infection, approximately 5-20% of cells infected with each of the viruses remained viable.

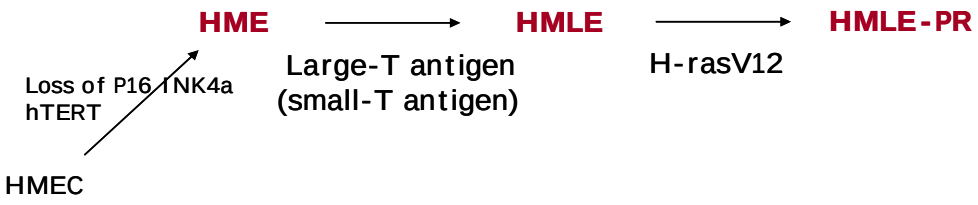
Figure 2: Nontumorigenic MCF-10A cells are more resistant to killing by rM51R-M virus than human and mouse breast cancer cells.



Task 2: Determine the susceptibility of breast cancer cells at different stages of tumorigenesis to infection with VSV.

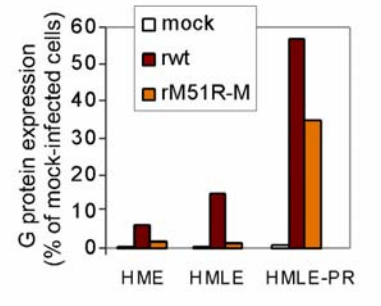
Our hypothesis is that during tumorigenesis, cells acquire defects in their antiviral responses that render them susceptible to infection with VSV. Therefore, as shown in figures 1 and 2, whereas normal cells are resistant to VSV, tumor cells are more sensitive. To further elucidate this point, we utilized mammary cells that are in different stages of tumorigenesis (6). Weinberg's group developed several transformed cell lines by the introduction of specific cancer-associated genes into human mammary epithelial cells (HMECs) as shown in Figure 3. Studies showed that the tumorigenicity of these cells was dependent on the level of ras oncogene expression. Therefore, these cells represent a powerful tool to determine how cancer cells at different stages of tumorigenesis respond to infection with VSV.

Figure 3: Human Breast Cancer Cells Generated by Oncogenic Transformation of HMEC



For these experiments, HME, HMLE (large T antigen) and HMLE-PR (large T antigen and H-Ras) cells were infected with rwt or rM51R-M viruses for 6 hours at a multiplicity of 10 pfu/cell. Cells were incubated with antibodies to the viral G protein and surface expression of G protein was determined by flow cytometry (Figure 4). We can see that HMLE-PR cells are more sensitive to VSV infection than the HME and HMLE cells as indicated by greater surface expression of G protein. This result suggests that as cancer cells acquire greater mutations for growth advantage, they also acquire mutations rendering them more susceptible to infection with VSV.

Figure 4: Human mammary cells transformed with SV40 large-T antigen and H-Ras oncoprotein are more susceptible to VSV infection than normal mammary cells.



In summary, these initial studies show that while breast cancer cells are sensitive to VSV-induced cytopathic effect, normal mammary cells are more resistant to the virus. Furthermore, they show that as cells become more tumorigenic, they may acquire additional mutations in antiviral responses rendering them more susceptible to VSV. These results are similar to those obtained in the prostate system, where normal prostatic epithelial cells obtained from prostatectomy patients are more resistant to infection and killing by VSV than the prostate cancer cell line, LNCaP (1). However, we have shown that prostate cancer cells are differentially susceptible to VSV infection. Therefore, it is important to note that not all cancer cells contain defects in antiviral pathways rendering them sensitive to VSV infection. Therefore, cancer cells may evolve to be inherently resistant to treatment with a variety of therapeutic agents, including VSV. In addition, it is possible that the immune response may also alter the ability of VSV to target and kill tumor cells in vivo. Therefore, Aim 2 of this proposal addresses these concerns and seeks to determine whether the effectiveness of VSV treatment can be enhanced by co-treatment with the anti-tumor cytokine, IL-12.

Aim 2: Determine the ability of VSV, together with IL-12, to treat breast cancer in vivo.

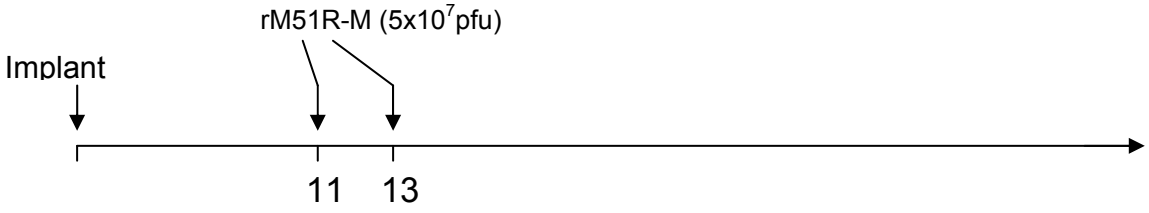
Task 1: Determine the role of the immune response during anti-tumor therapies with VSV. This will be done by implanting 4T1 cell in the flanks of athymic nude mice and immuno-competent mice and treating them intratumorally with rM51R-M virus.

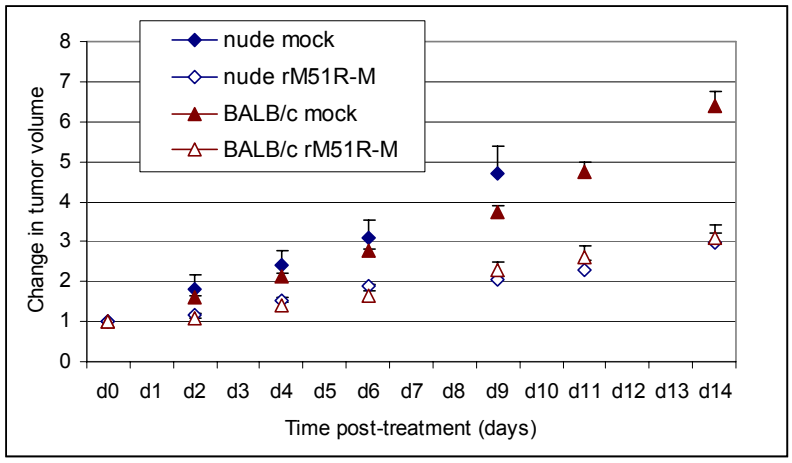
For these experiments, $5x10^5$ 4T1 cells were implanted in the flanks of BALB/c nu/nu and BALB/c wt mice (5 mice per group). When palpable tumors had formed, they were treated with rM51R-M virus intratumorally at day 11 and 13 post-implantation or mock-treated. 5 mice were included in each group. Each treatment provided $5x10^7$ pfu of virus at the tumor site. The tumor volume and weight of mice was measured 3 times/week. The health status of the mice was

monitored daily. Animals exhibiting signs of lethal VSV infection, such as hind limb paralysis, excessive tumor burden, or other signs of illness were euthanized as indicated by ACUC guidelines of Wake Forest University Health Sciences.

Figure 5 shows the fold change in tumor volume (as measured by the formula w^2 x L/2, where w=width of tumor and L=length of tumor) from the time of the initial treatment. The tumors of mock-treated nude animals showed uncontrolled growth over time and had to be euthanized by day 10 post-treatment due to excessive tumor burden. In contrast, nude mice treated with VSV showed decrease in rate of tumor growth indicating that rM51R-M virus was effective at delaying rate of tumor growth. BALB/c wt animals that were mock-treated also exhibited uncontrolled growth of tumor, but did not reach levels seen in the mock-treated nude animals. These data illustrate that the immune response does play some role in decreasing growth of the tumor. In addition, although the tumor volumes of BALB/c wt animals treated with rM51R-M virus decreased to the same levels as that seen with rM51R-M treated nude animals, the difference from that of mock-treated wt animals was not very great. These pilot experiments suggest that the immune response does control growth of the tumor and may play a role in decreasing the effectiveness of rM51R-M virus therapies in these animals, perhaps by attenuating virus infection and spread. Further experiments that are beyond the scope of this grant seek to determine whether specific immune components contribute to tumor therapies using VSV.

Figure 5: Treatment of 4T1 tumors in BALB/c wt and nude animals



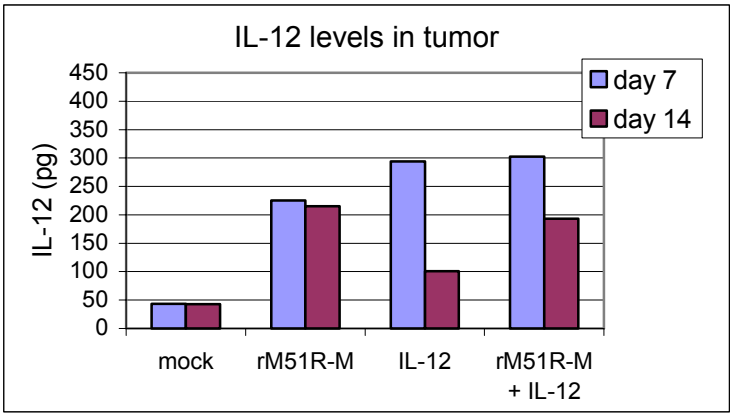


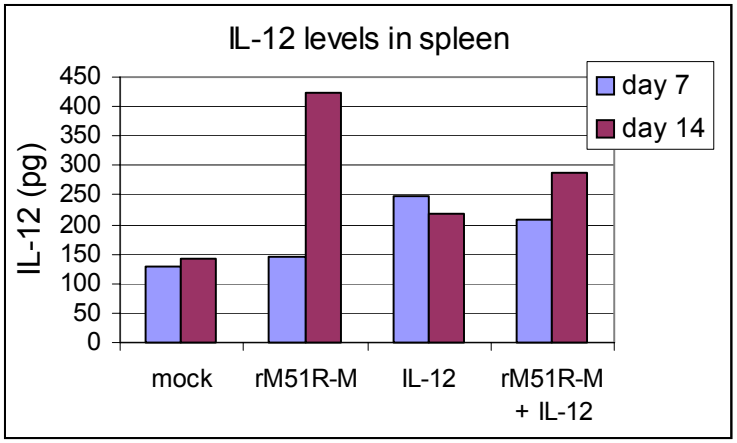
Task 2: Determine the effectiveness of rM51R-M virus therapies combined with IL-12 for the treatment of breast cancer in vivo.

For these experiments, we implanted 4T1 cells into the flanks of BALB/c mice as before. When palpable tumors had formed, we divided animals randomly into 4 groups of 5 mice. The experiment was carried out 2 times for a total of 10 mice per group. Group 1 was mock-treated, Group 2 was treated with rM51R-M virus at day 11 and 13 post-implantation. Group 2 was treated with 50ug of IL-12 naked DNA at days 11, 13 an 15 post-implantation. Group 4 received treatment with both rM51R-M and IL-12 according to the respective schedules. As before, tumor volumes, weights of animals, and signs of illness were monitored consistently. In addition, we determined the levels of IL-12 and IFNγ cytokines at the tumor site and spleen, and the presence of lung metastases.

Figure 6 shows the levels of IL-12 cytokine 7 and 14 days after the first treatment. For this experiment, tumors and spleen were homogenized and the supernatant was collected for standard ELISA reactions. Data was normalized for total weight of tissue and expressed in pg.

Figure 6: IL-12 levels in tumor and spleen at 7 and 14 days post-treatment.



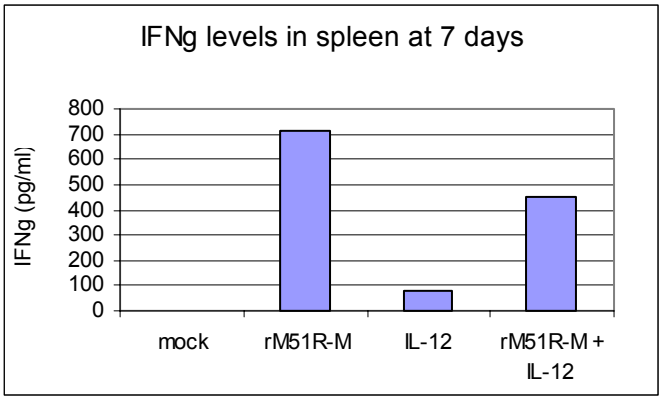


Results show that IL-12 levels increased at the tumor site at 7 days following each of the treatments. Interestingly, rM51R-M virus alone was capable of inducing expression of IL-12

cytokine at the tumor site upon treatment. The level of IL-12 induced by rM51R-M virus was similar to that stimulated by IL-12 alone and IL-12 + rM51R-M virus treatments. Furthermore, at day 14 post-treatment, results show that a sustained level of IL-12 was only observed upon treatment with rM51R-M virus. There may be a slight advantage to the combination treatment in the expression of IL-12 levels in the tumor, but further experiments need to be carried out to obtain statistical significance.

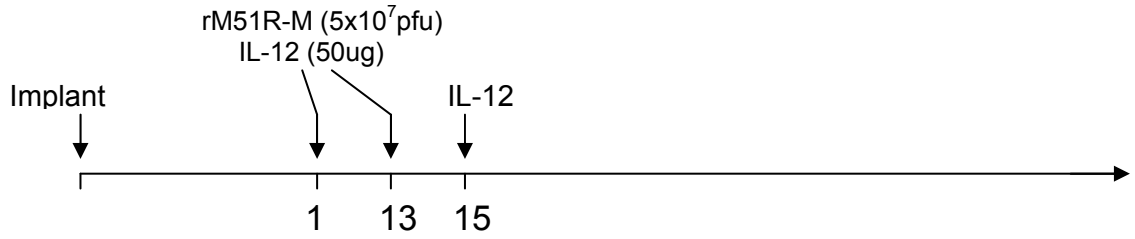
In the spleen, it appeared that early expression of IL-12 occurred following treatment with IL-12 cytokine expressing plasmid, while rM51R-M virus induced IL-12 expression in this tissue at later times post-treatment. Overall, these data indicate that rM51R-M virus is able to effectively induce expression of IL-12 at both local and distal sites. Furthermore, combination therapy aids in inducing an early burst of IL-12.

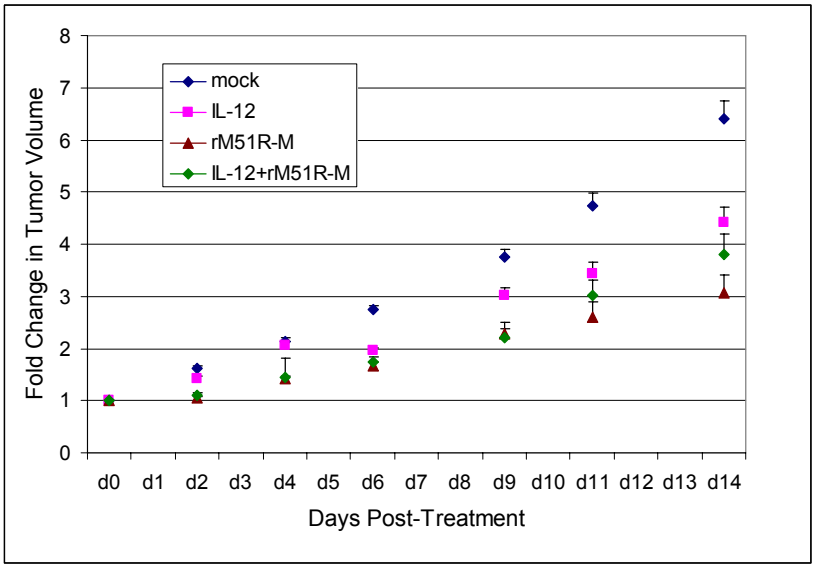
Figure 7: IFNγ levels in spleen at day 7 post-treatment.



In contrast to IL-12, expression of IFNγ was induced by rM51R-M virus at 7 days post-treatment at levels that were higher than those in IL-12 treated animals (figure 7). These data, together with IL-12 data suggest that rM51R-M virus is able to induce both an effective innate and perhaps adaptive immune response. However, further studies must be carried out to determine the ability of IL-12 cytokine to contribute to the induction of tumor specific immune responses.

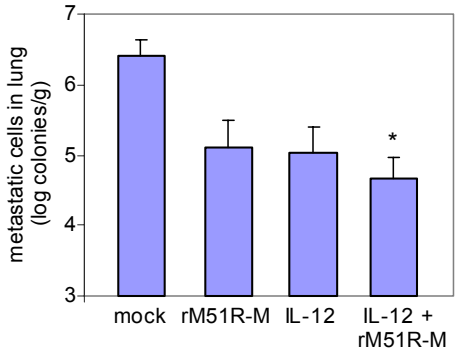
Figure 8: Change in tumor volume following treatment with rM51R-M virus and IL-12.





Data in figure 8 indicate that each of the therapies delayed the growth of the tumor in vivo as compared to the tumors, in mock-treated animals (statistically different from mock at day 14 post-treatment). Interestingly, the combination of rM51R-M virus together with IL-12 did not produce a more dramatic result as compared to rM51R-M virus alone or IL-12 alone.

Figure 9: Metastatic colonies in lung.



To determine whether local treatment affects metastases, we determined the number of metastatic cells in the lung via a clonogenic assay in the presence of 6-thioguanine (4T1 cells are resistant to 6-thioguanine). Interestingly, results in figure 9 showed that there was a decrease in number of metastatic colonies in the lungs of animals that were treated with any combination of rM51R-M virus or IL-12. In fact, the combination therapy was most effective at decreasing the metastases of these cells to the lung.

KEY RESEARCH ACCOMPLISHMENTS:

- VSV, specifically rM51R-M virus, kills tumor cells while sparing normal cells
- Cells at later stages of tumorigenesis acquire mutations that render them more susceptible to infection with VSV.
- The immune response serves to limit growth of aggressive 4T1 tumors.
- rM51R-M virus decreases tumor volume in BALB/c nude mice as well as in immunocompetent animals. Therefore, in this model, the immune response does not play a role in delaying tumor growth upon treatment with rM51R-M virus.
- rM51R-M virus alone, or IL-12 cytokine alone, is as effective as the combination therapy at delaying primary tumor growth in vivo.
- The combination therapy may be more effective than single treatments at delaying growth of metastases.

REPORTABLE OUTCOMES:

Abstract: Department of Defense Breast Cancer Research Program Meeting, 2005. Sensitivity of Breast Tumors to Oncolytic Viruses.

Seminar: Breast Cancer Center of Excellence, Wake Forest University Health Sciences, 2006. Treatment of Breast Cancer with Oncolytic VSV.

CONCLUSIONS:

These results indicate that treatment with the M protein mutant virus, rM51R-M virus, is as effective as IL-12 in the partial treatment of breast cancer and lung metastases when administered locally. Interestingly, rM51R-M virus alone induces the expression of IL-12 cytokine to levels comparable to the IL-12 plasmid treatment. Furthermore, combination therapy did not appear to provide additional advantage in the tumor, but may have some added benefit in the treatment of distal metastases. These results indicate that boosting the immune response with either an oncolytic virus that activates the immune response or a cytokine that produces tumor specific T cell responses provides a better therapy for a tumor type that is poorly immunogenic and may have mechanisms to suppress immune responses. Nevertheless, as shown by the data, although these therapies delay tumor growth, none of these therapies are able to completely eliminate the existing tumor. It is possible that further enhancing the immune system may be helpful in overcoming some of those suppressive tumor mechanisms. Alternatively, it is possible that boosting the oncolytic activity of the virus may be more effective at eliminating the tumor than enhancing the immune response. Future work will include further determining the nature of the immune response upon therapies with rM51R-M virus. Upon completion of these studies, we will be able to better understand the role of the immune response in treatment of this particular type of aggressive breast cancer model and perhaps use this information to design more effective viruses and combination therapies for treatments of aggressive cancers.

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